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Components of both major axial patterning systems of the Bilateria are differentially expressed along the primary axis of a 'radiate' animal, the anthozoan cnidarian *Acropora millepora*

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Abstract

Cnidarians are animals with a single (oral/aboral) overt body axis and with origins that nominally predate bilaterality. To better understand the evolution of axial patterning mechanisms, we characterized genes from the coral, *Acropora millepora* (Class Anthozoa) that are considered to be unambiguous markers of the bilaterian anterior/posterior and dorsal/ventral axes. Homologs of Otx/otd and Emx/ems, definitive anterior markers across the Bilateria, are expressed at opposite ends of the *Acropora* larva; *otxA-Am* initially around the blastopore and later preferentially toward the oral end in the ectoderm, and *emx-Am* predominantly in putative neurons in the aboral half of the planula larva, in a domain overlapping that of *cnox-2Am*, a Gsh/ind gene. The *Acropora* homologs of Pax-3/7, NKX2.1/vnd and Msx/msh are expressed in axially restricted and largely non-overlapping patterns in larval ectoderm. In *Acropora*, components of both the D/V and A/P patterning systems of bilateral animals are therefore expressed in regionally restricted patterns along the single overt body axis of the planula larva, and two 'anterior' markers are expressed at opposite ends of the axis. Thus, although some specific gene functions appear to be conserved between cnidarians and higher animals, no simple relationship exists between axial patterning systems in the two groups.

Keywords: Axial patterning; Homeobox genes; Coral; Anthozoa; Cnidaria

Introduction

One of the outstanding questions in animal biology is the origin of bilaterality–how, and from what ancestral morphology, the mirror image symmetry of the Bilateria arose. Conserved molecular mechanisms underlie many aspects of the specification and patterning of both major body axes throughout the Bilateria, but although these systems are well understood in *Drosophila* and mouse, their evolutionary origins are equivocal (Holland, 2000; Martindale et al., 2002; Martindale, 2005). As the sister group to the Bilateria (Medina et al., 2001), the Cnidaria are likely to be particularly informative with respect to the origins of bilaterian patterning mechanisms. Homologs of many genes that play key roles in bilaterian patterning are known from various cnidarians (reviewed in Ball et al., 2004 and Martindale, 2005), but the canonical Hox system is likely to have arisen after the Cnidaria/Bilateria divergence (Kamm et al., 2006), so that at present, what has effectively been the Rosetta Stone of developmental genetics cannot be applied to the interpretation of cnidarian patterning systems.

To better understand the relationship between the single overt (the oral/aboral, or O/A) axis of cnidarians and the two (dorsal/ventral, or D/V, and anterior/posterior, or A/P) axes of

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bilaterians, we characterized a suite of genes in the anthozoan *Acropora millepora* that are considered to be unequivocal markers of the A/P and D/V axes. Previous attempts to address the origins of axial patterning systems have relied on single classes of gene, often in derived species, and comparisons between cnidarians are complicated both by the diversity within the phylum (http://www.ucmp.berkeley.edu/cnidaria) and the presence of paralogs of some key regulatory genes (reviewed in Ball et al., 2004). Whereas until recently the freshwater hydrozoan *Hydra* was the most widely studied cnidarian, anthozoans – members of the basal cnidarian Class Anthozoa, which includes the sea anemone *Nematostella* and the coral *Acropora* – have more faithfully maintained some ancestral characteristics (Bridge et al., 1992, 1995), and the available evidence suggests that this conservation extends to the ancestral gene set (Ball et al., 2004; Kusserow et al., 2005; Technau et al., 2005). For example, whereas homologs of the homeobox gene *even-skipped* (Miller and Miles, 1993) and the signaling molecule hedgehog are present in both *Nematostella* and *Acropora* (Technau et al., 2005), preliminary analyses of the draft genome indicate that both of these genes have been lost from *Hydra*. Moreover, some anthozoans display "biradial" symmetry (a form of bilaterality), and true radial symmetry is seen only in derived members of the phylum. In *Acropora* (Hayward et al., 2002, Ball et al., unpublished) and *Nematostella* (Finnerty et al., 2004), expression data for Dpp/BMP4 homologs are consistent with the view that some of the mechanisms underlying bilaterality may predate the Cnidaria/Bilateria split, although the roles of these molecules in cnidarian patterning are unknown.

What genes should one use to investigate the conservation of mechanisms of axial determination? As characteristic anterior genes, we chose to investigate early developmental expression patterns for the *Acropora* homologs of the *Drosophila* head gap genes *otd* and *ems*. An extensive literature documenting the evolutionary conservation of these genes has developed since the recognition of conserved expression patterns in the heads of flies and mice by Simeone et al. (1992). Although these genes are mainly neurally expressed, they are highly conserved anterior markers (reviewed by Lichtneckert and Reichert, 2005). Indeed, Otx and Emx are such faithful and universal markers that Slack et al. (1993) proposed their inclusion (with the Hox genes) in the "zootype," the universal genetic toolkit of animals. Among the Cnidaria, Otx expression has only been characterized in the hydrozoans *Podocoryne* (Mueller et al., 1999), where it is initially expressed in medusa buds and becomes restricted to striated muscle, and *Hydra* (Smith et al., 1999), where it is expressed at high levels in developing buds and lower levels throughout the column. The only cnidarian Emx for which expression data are available is that of *Hydractinia* (Mokady et al., 1998), discussed in more detail below.

While BMP2/4 (*Drosophila* ortholog *dpp*) and its antagonist chordin (*Drosophila* ortholog *sog*) appear to be good D/V markers across the Bilateria, in *Nematostella* they are co-localised, leading Matus et al. (2006) to hypothesize that their primitive function was in establishing germ layer identity or a boundary for epithelial patterning. There is, however, another set of dorso/ventral patterning genes which is phylogenetically conserved. This is *vnd*, *ind* and *msh*, which are involved in patterning the nervous systems of flies and vertebrates (Cornell and von Ohlen, 2000). Gsh/ind genes have been identified in representatives of three of the four cnidarian classes (reviewed in Schierwater et al., 2002; Ball et al., 2004). Msh-related genes have been cloned from *Hydra viridissima* and *Hydra vulgaris* (Gauchat et al., 2000; Schummer et al., 1992) and a vnd gene from *Nematostella vectensis* (NK-2) is in Genbank (accession #AAP88430), but expression data are not yet available for these genes. We have previously shown that the *Acropora ind* homolog *cnox-2Am* is expressed in an axially restricted manner during development (Hayward et al., 2001), hence expression data for vnd and msx genes are of particular interest. We also studied the early expression pattern of the *Acropora Pax-Dam* gene (Miller et al., 2000), since Pax-3/7-class genes also have key roles in D/V patterning of the CNS across the Chordata (Mansouri and Gruss, 1998; Wada et al., 1997). We report that all of these genes are differentially expressed along the single overt axis during early *Acropora* development, in patterns that are reminiscent of those of Hox genes in insects and mammals, suggesting that in the absence of a canonical Hox cluster (Kamm et al., 2006) analogous roles might be fulfilled by homeobox genes with different DNA-binding specificities. Although comparisons with higher animals are complicated by the presence of paralogs of several of these genes, these studies, together with those of Matus et al. (2006) clearly demonstrate that no simple relationship exists between the single overt axis of cnidarians and either of the bilaterian axes.

Materials and methods

Fixation and storage

A. millepora embryos and larvae representing all of the major morphological stages of development were fixed for 15–60 min in 3.7% formaldehyde in Millipore-filtered seawater (MPFSW) buffered to pH 8.0 with HEPES buffer. Fixed material was then washed repeatedly in MPFSW, dehydrated through a graded methanol series and stored in absolute methanol at −20°C until needed.

Whole mount in situ hybridization

Probe production and in situ hybridization were carried out as described in Hayward et al. (2001), using either BM Purple AP Substrate (Roche) or BCIP/NBT Alkaline Phosphatase Substrate Kit IV (Vector) as substrate. The former was generally used for mapping the distribution of expressing cells, and the latter for observing cellular morphology. For the respective genes, probes consisted of the anti-sense strand of the entire sequence supplied to Genbank, hydrolyzed to approximately 400 bp. Some genes, in particular Otx (Fig. 1) and Emx (Fig. 2) apparently show considerable expression in the endoderm. However, since most of the endoderm of the planula consists of large cells filled with lipid and very little cytoplasm, we interpret this staining as artifactual. The appearance of endodermal staining is also accentuated because in cleared specimens diffuse color comes through from the ectoderm on the other side of the embryo, which underlies the endoderm and because the cylindrical shape of the planula means that a uniform background stain actually appears stronger at the center of the cylinder.

Antibody staining

Embryos were gradually rehydrated from 100% MeOH to PBS containing 0.2% Triton X (PBS-Tx). After repeated washes, fixed tissues were pre-blocked in 5% normal goat serum (NGS) in PBS-Tx and then incubated with a 1:500 or

1:1000 dilution of rabbit anti-FMRF amide (Peninsula Labs, IHC 8755). After 12–36 h at 4°C, the tissues were washed for 2–3 h in at least 4 changes of PBS-Tx and then incubated (2 h at RT or overnight at 4°C) with a Cy-5 labeled goat anti-rabbit IgG (Jackson Immunoresearch) at 1:200. After approximately 2 h of washing with at least 4 changes of PBS-Tx, the embryos were counterstained with Hoechst (bis-benzimide) (Sigma) at 1:1000 in the second last change of PBS-Tx.

Clearing and photography

In situ preparations were dehydrated through a graded series of glycerol solutions and mounted in 90% glycerol. Photographs were taken using a Zeiss Axioskop or a Wild Photomakroskop. Images were captured on Kodak Ektachrome 64 tungsten film and then scanned or directly captured in digital form using QImaging or Spot digital cameras. Antibody-stained preparations were viewed and photographed on a Deltavision Deconvolution microscope. Digitized images were processed using Adobe Photoshop.

Sequence and phylogenetic analyses and their presentation

Maximum likelihood phylogenetic analyses were conducted using MolPhy version 2.3 (Adachi and Hasegawa, 1996) using the Dayhoff substitution matrix and local rearrangement search mode. In the phylogenetic trees shown as part B of Figs. 1–5, numbers on nodes in the trees represent the percentage of 1000 bootstrap replicates supporting the topology shown. Numbers to the right of sequences in part A of these same figures indicate percent identity/similarity to the *Acropora* sequence, or to the first sequence listed where multiple *Acropora* sequences are included. Sequences are arranged in Boxshade in the following order; other cnidarians, deuterostomes and protostomes. In the figures, red is used to identify *Acropora* sequences, and where corresponding *Nematostella* sequences could be identified by analyses of the trace archive data at NCBI, these are shown in blue. Sequences are designated by gene names and genus/species abbreviations. The latter include: Am, *Acropora millepora*; Bf, *Branchiostoma floridae*; Ce, *Caenorhabditis elegans*; Ci, *Ciona intestinalis*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio*; Es, *Euprymna scolopes*; Gg, *Gallus gallus*; Hp, *Holopneustes purpurescens*; Hp, *Hemicentrotus pulcherrimus*; Hr, *Halocynthia roretzi*; Hs, *Homo sapiens*; Hsy, *Hydractinia symbiolongicarpus*; Ht, *Helobdella triserialis*; Hvi, *Hydra viridis*; Hvu, *Hydra vulgaris*; Lj, *Lethenteron japonicum*; Ls, *Lineus sanguineus*; Mm, *Mus musculus*; Nv, *Nematostella vectensis*; Pc, *Podocoryne carnea*; Pm, *Phallusia mammilata*; Pv, *Patella vulgata*; Sc, *Scyliorhinus canicula*; Sp, *Strongylocentrotus purpuratus*; Xl, *Xenopus laevis*; Xt, *Xenopus tropicalis*. For clarity, a common format was adopted for gene names in the figures; names as they appear in Genbank, together with accession numbers, are listed in the Supplementary material.

Results

Two genes related to the otd/Otx gene in Acropora

Two otx sequences were identified from *Acropora* adult ESTs. Two related genes were identified in *Nematostella*; one is in the databases as AAR24459 (*otx1-Nv*), and the second (*otx2-Nv*) was recovered from the trace archive at NCBI. None of the anthozoan proteins contains convincing matches to the

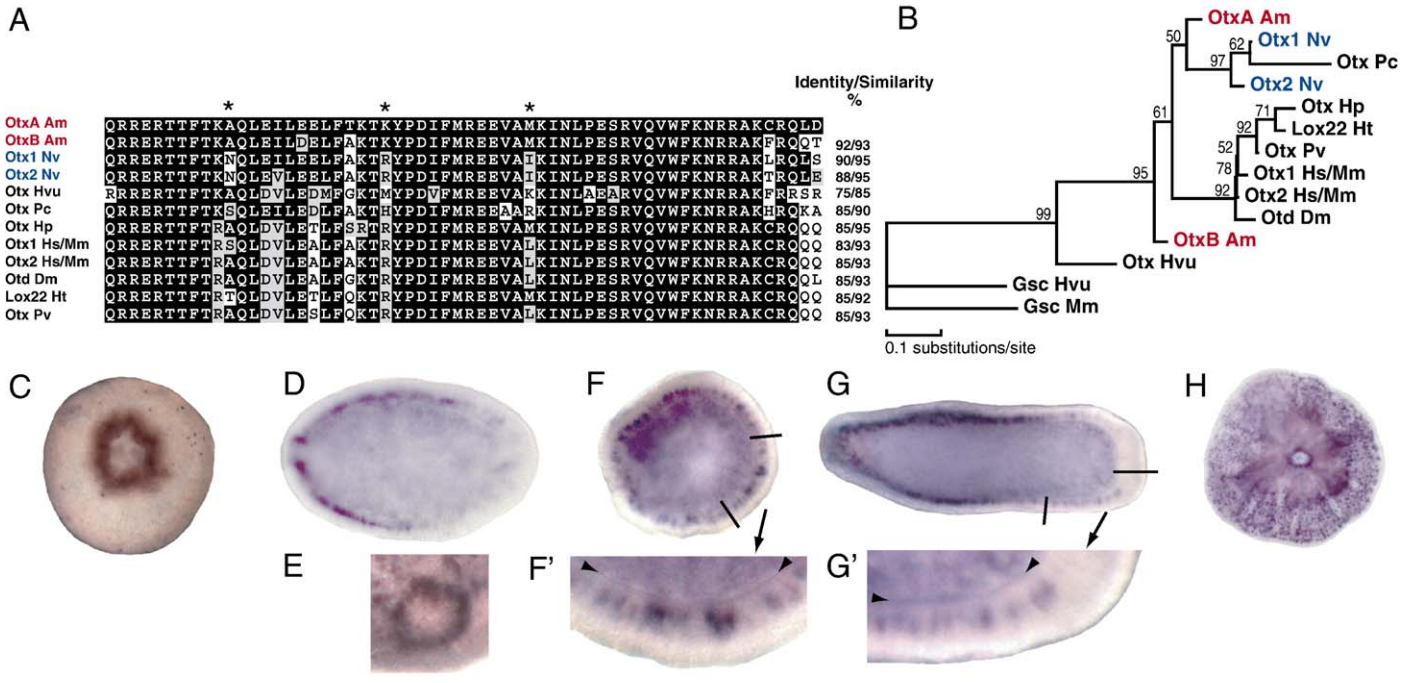


Fig. 1. Phylogenetic analysis and expression of *otxA-Am*. (A) Alignment of the 60 amino acid homeodomains encoded by the two *Acropora* Otx genes with those of related proteins. Asterisks above the sequences indicate residues that are identical in the two proteins in both *Acropora* and *Nematostella*, but differ between species, suggesting independent duplication or concerted evolution. (B) ML phylogenetic analyses of the sequences shown in part A, using the *Mus musculus* and *Hydra* Gsc sequences as outgroups, imply that relationships between the *Acropora* and *Nematostella* Otx sequences are not simple. (C–H) Expression data for *otxA-Am*. In planulae, the oral pore is to the left and the aboral end, which is anterior in the swimming animal, is to the right. In gastrulating and post-settlement specimens, the oral pore faces the reader unless otherwise noted. (C) Expression is first clearly apparent in the ectoderm surrounding the blastopore. (D–E) Strong expression is retained around the oral pore (D, E) of the early planula and appears in the basal portion of the ectoderm just above the mesogloea (F'). (F) In this transverse section of a planula, the strongest expression is in scattered cells in the ectoderm, as is clearly apparent in panel F' where arrowheads mark the position of the mesogloea. In panel G, it appears that this population of cells is distributed even beyond the oral two-thirds of the planula where it stains most intensely. The higher magnification view shown as panel G' again clearly shows that the expression is ectodermal (arrowheads mark the mesogloea). (H) Post-settlement, both the oral staining and the staining in ectodermal cells are retained, although the latter appear more scattered than in the planula.

WSP motif that is present in some otx homologs, including *Podocoryne Otx-Pc*. Phylogenetic analyses indicate that relationships between the *Acropora* and *Nematostella* Otx genes are not simple, i.e. direct orthology relationships are unlikely (Fig. 1B). Three positions within the homeodomain (residues 11, 24 and 36; indicated by asterisks in Fig. 1A) are the same in both proteins from each species but differ between the species, suggesting either the possibility of independent duplication events since the divergence between corals and sea anemones or that the loci might evolve in concert. However, the relative positions of the hydrozoan and anthozoan sequences in the tree shown as Fig. 1B imply that otx genes may have a more complex history in cnidarian evolution.

The earliest visible expression of *otxA-Am* is in the ectoderm surrounding the closing blastopore (Fig. 1C). This expression is weaker than that occurring later in development. As the embryo elongates from a spherical gastrula into a pear shaped planula strong expression continues around the oral pore (Figs. 1D, E) and expression becomes more widespread (Fig. 1D) concentrated in a population of ectodermal cells that extend only about halfway across the ectoderm (Figs. 1D, G). It appears that this population of cells occurs throughout the embryo, but does not stain strongly in the aboral one third (e.g. Fig. 1G). Post-settlement expression is in a population of scattered ectodermal cells and continues in the ectoderm surrounding the mouth (Fig. 1H). By contrast, expression of *otxB-Am* is not axially restricted; *otxB-Am* is expressed in the endoderm throughout development (data not shown).

An ems/Emx gene in Acropora

A single emx gene was identified in *Acropora*, and a probable ortholog (*emx3-Nv*) was retrieved from the *Nematostella* raw genomic sequence data. In addition, two other related sequences were also identified in *Nematostella* (Figs. 2A, B), one of which (*emx2-Nv*) is a likely paralog of *emx-Am/emx3-Nv*, whereas the other (*emx1-Nv*) is highly divergent. The predicted Emx-Am protein contains an Emx/ems-type homeodomain near the N-terminus which most closely resembles those of lamprey, dogfish and *Xenopus* Emx proteins (82% identity; see Fig. 2A). Despite slightly lower identity with the *Hydractinia* Cn-ems (Ems Hsy in Figs. 2A and B) homeodomain (78%), these cnidarian sequences grouped together in phylogenetic analysis and were well resolved from all of the bilaterian Emx sequences (Fig. 2B). The Emx-Am protein does not contain a convincing hexapeptide motif—the closest candidate is the SFYPCA motif at amino acids from −33 to −28 relative to the homeodomain.

In situ hybridization revealed that *emx-Am* is first expressed as the oral–aboral (O/A) axis becomes apparent in early "pear" stage planulae in a subset of transectodermal cells that are restricted in their distribution to the aboral and central regions of the planula larva (Figs. 2C, D). Cells containing this message are always absent from the oral end. Two clearly distinct emx-Am expressing cell types are observed in these stages. The first cell type is tri- or multipolar with the nucleus and bulk of its cytoplasm located close to the mesogloea (Figs. 2F1, F2, upward pointing black arrows). Cellular extensions along the mesogloea are often apparent (Figs. 2F1, F2). Each cell also has a fine projection toward the surface of the ectoderm. The second cell type is thin and bipolar with a nucleus located midway across the ectoderm (Fig. 2F3, upward pointing white arrow). One sometimes sees *emx*-expressing cells without obvious extensions across the ectoderm (Figs. 2F4, F5). These may represent an additional cell type or they may be artifactual products of the first type arising from sectioning or incomplete staining. Given the known limitations of in situ hybridization for detecting fine neural projections, these cells fulfil all of the morphological criteria to be neurons and appear very similar to the RFamide-expressing neurons of *Acropora* (Figs. 2G, H). The staining associated with the base of the cells shown in Figs. 2F1 and F2 is consistent with the presence of axons extending along the mesogloea, as in the case of the RFamide-staining axons marked by white arrows in Fig. 2G. Although definitive proof is lacking, the emx-expressing cells clearly fit the published descriptions of cnidarian neurons (Grimmelikhuijzen and Westfall, 1995; Chia, 1979) but not other known ectodermal cell types (Fautin and Mariscal, 1991).

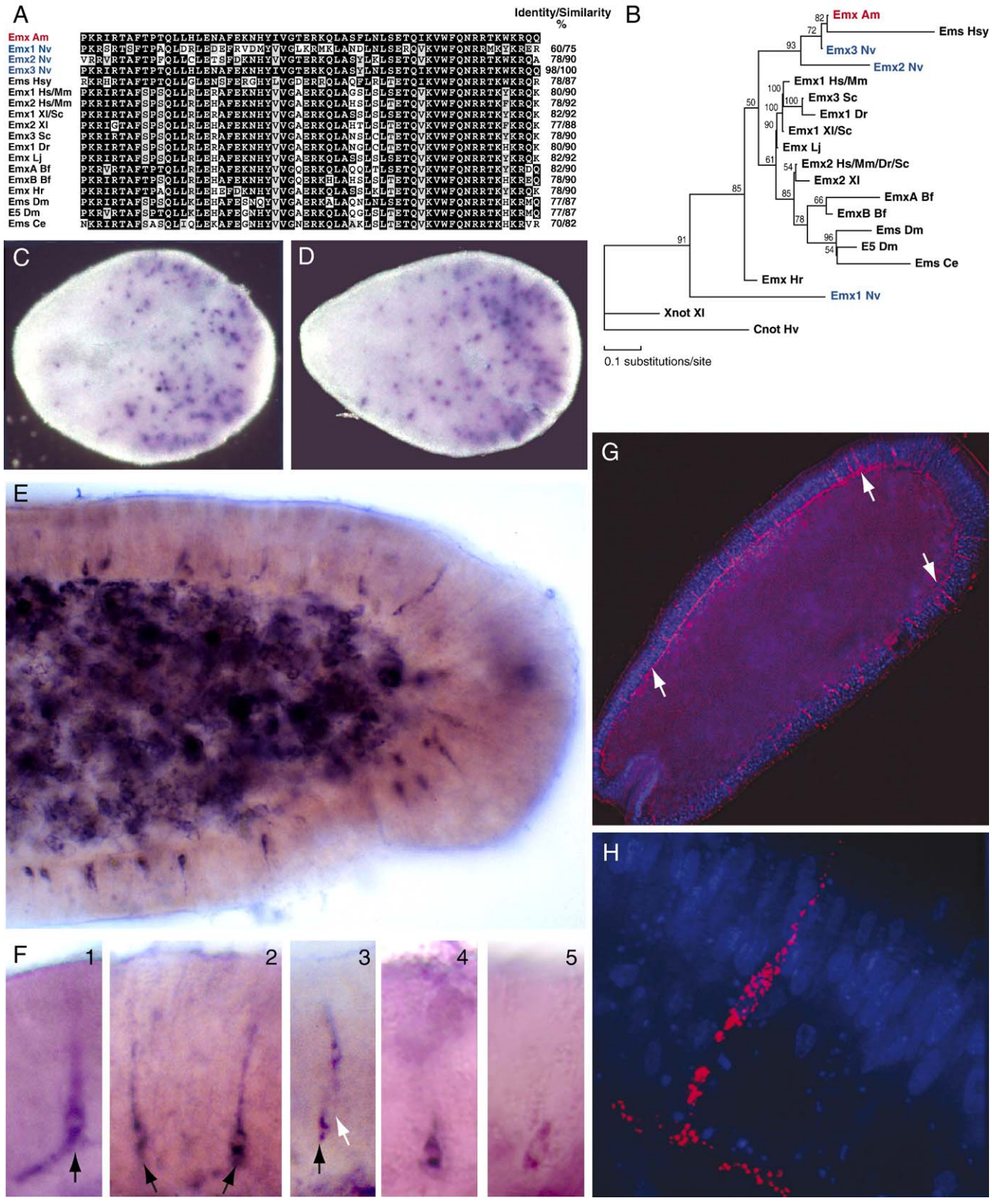
As the planula continues to develop, it lengthens and becomes thinner, extended and spindle-like in appearance. In some preparations at this stage, the number of *emx-Am* expressing transectodermal cells appears to be considerably reduced in the ectoderm. This may be due to a combination of factors. Firstly, other cell types may be proliferating disproportionately to neurons. Secondly, in late stage planulae, this disappearance might be associated with degeneration of the nervous system in preparation for settlement. This phenomenon has been documented in hydroids (Kroiher et al., 1990; Martin, 2000), and is apparent in *Acropora* when a series of stages spanning settlement is stained with anti-RFamide (J. Reece-Hoyes and E. Ball, unpublished).

The aboral expression of *emx-Am* in *Acropora* larvae corresponds to the anterior expression of related genes in bilateral animals in the sense that coral planulae swim aboral end first, and the expression in putative neurons is consistent with the known functions of related genes in Bilateria. This pattern of expression is very different to that of the only other cnidarian emx gene for which data are available; *Cn-ems* (Ems Hsy in Figs. 2A and B) from Hydractinia (Mokady et al., 1998). Cn-ems expression is only seen in adults or planulae induced to form adult structures and in the opposite end of the primary axis: it is restricted to the endodermal epithelial cells of the taeniolae of the hypostome in the head of gastrozooids. These differences, and the extent of sequence divergence, suggest the possibility that emx paralogs are being compared (but see Fig. 2B).

It is difficult to interpret the significance of the *emx-Am* expression pattern with respect to that of *cnox-2Am*, which is also expressed in putative neurons in the same axis and at approximately the same time during development in an axially restricted manner (Hayward et al., 2001). It is not yet clear whether these two genes are co-expressed. They may mark two distinct populations of cells as the number of RF-amide positive cells appears to be greater than the number of *emx-Am* cells in the central part of the planula.

Genes related to Vnd/Nkx2.1

cDNAs were identified corresponding to three distinct *Acropora* genes related to *Drosophila vnd* and vertebrate *Nkx2.1*. These cnidarian genes have been duplicated independently of their four vertebrate homologs (see Fig. 3B); phylogenetic analyses do not support orthology between any single cnidarian/vertebrate sequence pair. Two of the *Acropora* vnd loci are tightly linked in a 'head-to-head' orientation (Hislop et al., 2005), but the location of the third has not been



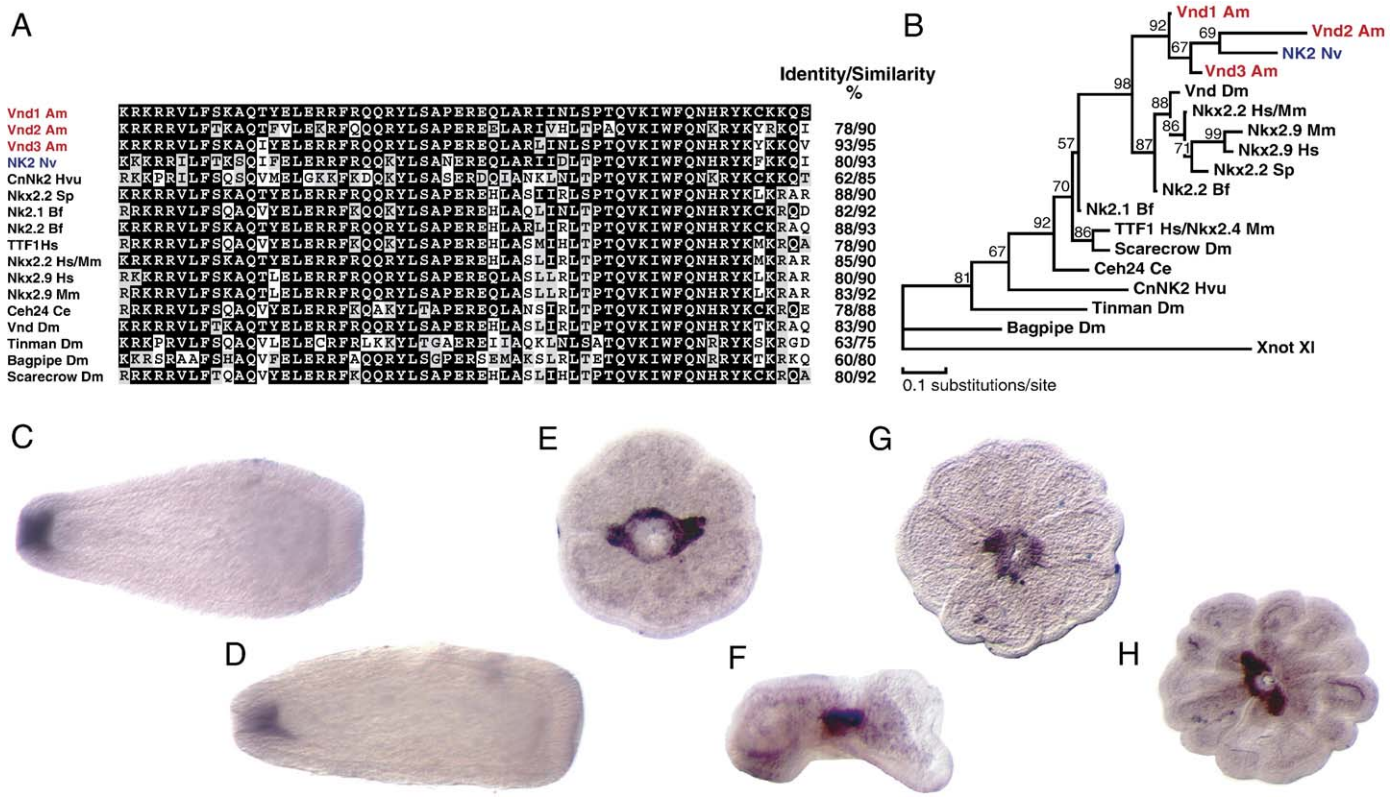


Fig. 3. Phylogenetic analysis and expression of vnd genes in *Acropora*. (A) Alignment of the 60 amino acid homeodomains present in the three *Acropora* vnd proteins and related sequences. (B) ML phylogenetic tree of the sequences shown in part A, using the *Xenopus laevis* Xnot sequence as outgroup. (C–H) Expression data for *vnd3-Am*; patterns were indistinguishable for the other two genes in the stages examined. (C–D) Planulae showing *vnd3-Am* expression in the invaginated ectoderm of the pharynx. The oral pore is to the left and the aboral end, which is anterior in the swimming animal, is to the right. (E–H) Successively older post-settlement specimens showing expression associated with the mouth, in a pattern that is commonly biradial (E, H), but is sometimes radially symmetrical (G). (F) Sectioned post-settlement specimen confirming expression in the invaginated ectoderm associated with the mouth.

established. In addition to homeodomains clearly of the NKX2.1 type (Fig. 3A), each of the predicted *Acropora* Vnd proteins contains an Eh1 domain near the N-terminus; both Vnd1-Am and Vnd3-Am also contain NK2-specific domains (Harvey, 1996) immediately C-terminal of the homeodomain. The homeodomains of Vnd1-Am and Vnd3-Am show 93/95% identity/similarity, and the corresponding figures for Vnd1-Am versus Vnd2-Am and Vnd2-Am versus Vnd3-Am are 78/90% and 75/88%, respectively. A single related *Nematostella* gene was identified; while its homeodomain has highest similarity to that of Vnd2-Am (Figs. 3A, B), it differs in containing an NK2-specific domain in addition to the Eh1 and homeodomain that are present in the protein products of each of these genes. This implies that, as in the case of the otx genes (see above), orthology relationships between the *Nematostella* and *Acropora* NK2 genes may not be simple. The single published *Hydra* NK2 (CnNK2 Hvu) sequence (Grens et al., 1996) is of the NKX2.5/tinman type, hence its relatively distant position in phylogenetic analyses (Fig. 3B).

The expression patterns of each of the *Acropora vnd* genes were indistinguishable in the stages examined. Expression is first detected at the planula larva stage in the oral ectoderm (Fig.

Fig. 2. Phylogenetic analysis and expression of *emx-Am* with a comparison to RFamide expression (A) Alignment of the 60 amino acid homeodomain encoded by *Acropora emx-Am* with those of related proteins. (B) ML phylogenetic tree of the sequences shown in part A, using the *Xenopus laevis* Xnot and *Hydra vulgaris* Cnot sequences as outgroups. Three Emx-related sequences are encoded by the *Nematostella* genome; one of these (Emx3-Nv) is the presumed ortholog of Emx-Am, whereas Emx2-Nv is a likely paralog and Emx1-Nv is only distantly related to these. (C–F) Expression data for *emx-Am*. In planulae, the oral pore is to the left and the aboral end, which is anterior in the swimming animal, is to the right. (C–D) Expression is first clearly observed in a subset of trans-ectodermal cells (not all ectodermal cells clearly extend across the ectoderm) as the spherical gastrula elongates to form a planula larva, and this pattern is maintained in the elongate planula right up to settlement. Panels E and F illustrate the two distinct morphologies of *emx*-expressing cells in *Acropora.* The more abundant type, identified by black arrows, has a cell body at the base of the ectoderm and a projection upward across the ectoderm, which – at least in some cases – reaches the surface. Below the cell body the cell gives off projections along the mesogloea. The second, smaller cell type, identified by white arrows, is bipolar and has a nucleus located halfway across the ectoderm. Cells whose morphology does not clearly fit into either of these types are shown in panels F4 and F5; these most likely reflect staining or sectioning artifacts, but could possibly correspond to another cell type. (E) Aboral end of a planula comparable in age to that shown in panel G. Note that in both cases the staining transectodermal cells are either missing or much less abundant than further orally in the ectoderm. (G, H) Planulae double stained with antibody to the neuropeptide RFamide (red) and DAPI for nuclei (blue) for comparison with the *emx*-expressing cells shown in panels E and F. White arrows in panel G identify the axonal projections along the mesogloea. Note especially the morphological similarities between the *emx*-expressing cells in panels F1 and F2 and the RFamide expressing cells in panels G and H.

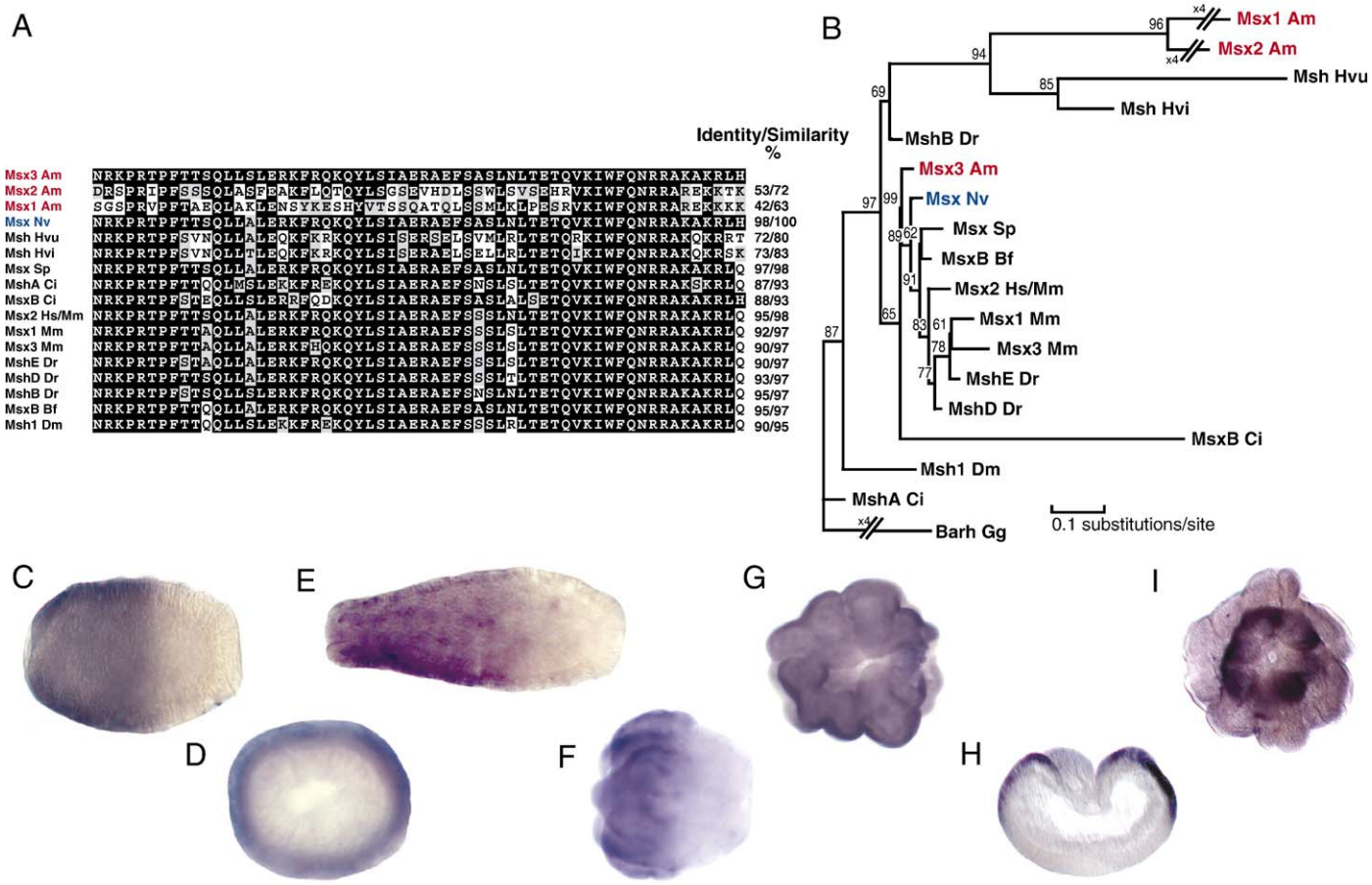


Fig. 4. Phylogenetic analysis and expression of *msx3-Am*. (A) Alignment of the 60 amino acid homeodomains encoded by the *Acropora* msx genes with those of related proteins. (B) ML phylogenetic tree of the sequences shown in part A, using the *Gallus gallus* BarH sequence as outgroup. Branches leading to Msx1-Am and Msx2-Am have been shortened; these homeodomains group with two published *Hydra* sequences and are clearly resolved from those of other Msx proteins, Msx3-Am and its presumed *Nematostella* ortholog Msx-Nv have higher levels of identity with bilaterian Msx proteins. (C–I) Expression data for *msx3-Am*. (C, E) Expression is localized to the oral two-thirds of the planula larva. (D) A transverse section of the staining portion of a planula reveals that expression is ectodermal. (F–H) During and following settlement expression is limited to the oral half of the embryo. (I) Oral expression is maintained in older post-settlement animals with expression strongest in the upward-growing polyp. The oral pore is to the left in panels C, E and F, out of the page in panels G, I and upward in H.

3C). As development proceeds, vnd expression is restricted to the invaginated ectoderm around the oral pore that forms the cup-like gastric cavity in pre-settlement embryos (Fig. 3D). During metamorphosis, the larva flattens to create the characteristic disc-shaped post-settlement form (Fig. 3E). Following metamorphosis, vnd expression is maintained in the ectoderm surrounding the oral pore (Fig. 3F), in a pattern that is sometimes symmetrical (Fig. 3G), but more frequently biradiate (Figs. 3E, H). At the oldest stage shown (Fig. 3H), expression is clearly biradiate; the two obvious arms of expression appear to coincide with the directive septa, and an obvious break occurs at right angles to these in the band corresponding to the ectoderm inside the mouth.

A functional Acropora msh/Msx gene

Previously we have described the identification of a pair of linked Msx/msh-related loci in *Acropora*, although these are likely to represent pseudogenes (Hislop et al., 2005). By applying a redundant PCR approach, we were able to identify a third Msx/msh gene in *Acropora*. Whereas the *msx1-Am* and *msx2-Am* sequences are relatively diverged, the *msx3-Am* cDNA encodes a protein highly similar to chordate Msx sequences. Searching the trace archives at NCBI allowed the identification of a clearly related gene in the *Nematostella* genome. The phylogenetic tree shown (Fig. 4B) indicates that, whereas the previously reported cnidarian Msx/msh-related genes formed a distinct clade irrespective of method of phylogenetic analysis, Msx3-Am and its presumed *Nematostella* ortholog group with the majority of deuterostome Msx/msh genes; note the corresponding branch lengths in Fig. 4B. The phylogenetic analyses imply that these two types of Msx/msh gene were distinct prior to the hydrozoan/anthozoan divergence, and that *msx-3Am* may more closely reflect the ancestral function(s) of the Msx/msh genes than previously known cnidarian representatives of this class. In addition to the homeodomain, which is most similar to that of the sea urchin *S. purpuratus* SpMsx (97/98% identity/similarity), the Msx3-Am protein is predicted to contain an Eh1 domain near the N-terminus and a motif resembling Msx-type

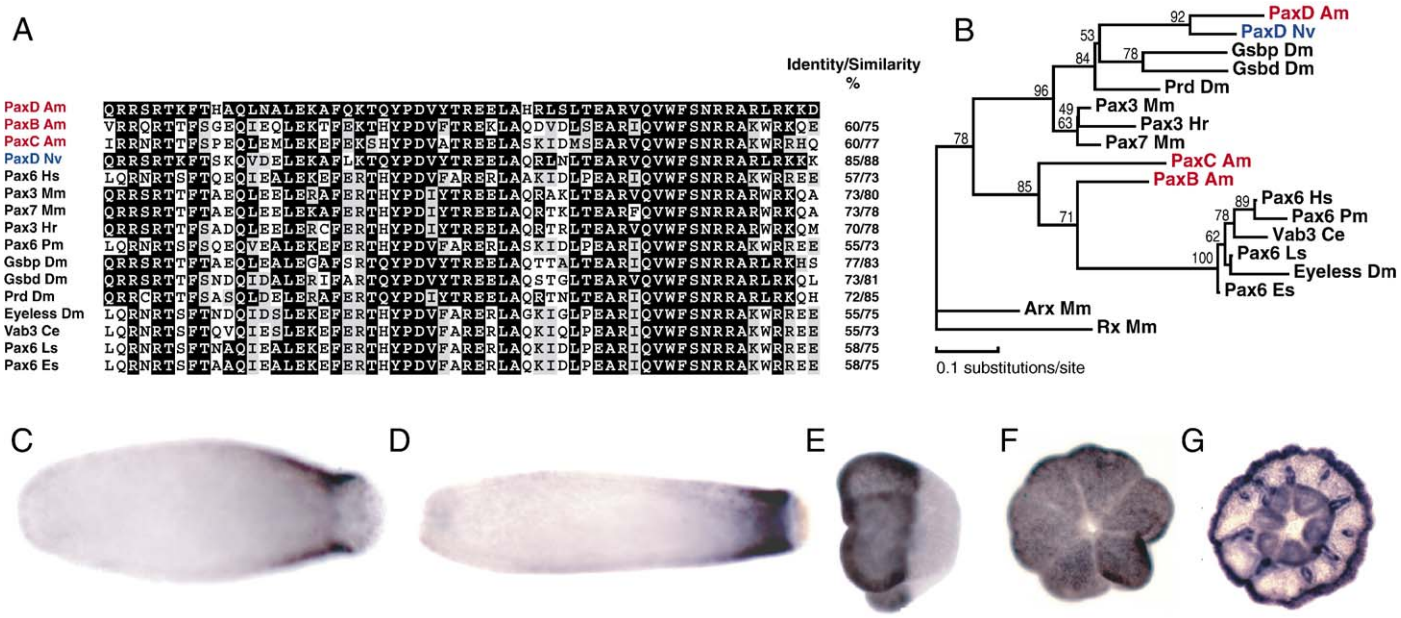


Fig. 5. Phylogenetic analysis and expression of *Pax-Dam*. (A) Alignment of the 60 amino acid homeodomain encoded by *Pax-Dam* with related sequences. The homeodomains of Pax-Dam and its presumed *Nematostella* ortholog PaxD-Nv are unambiguously related to the Pax-3/7 type (which includes *Drosophila* Prd and Gsb), whereas all other cnidarian Pax proteins are of the Pax-2/5/8/6-type. (B) ML phylogenetic tree of the sequences shown in part A, using the mouse Arx and Rx sequences as outgroups. (C–G) Expression data for *Pax-Dam*. (C, D) Expression is initially limited to a band of ectoderm covering anywhere from 1/4 to 1/6 of the planula at, but not extending across, the aboral end of the planula. (E, F) On settlement there is a dramatic reversal of the expression pattern, with the oral end of the polyp expressing to the exclusion of the aboral end. (G) An older polyp shows expression around its rim and in the endoderm associated with the medial portion of each septum.

hexapeptides around the FPWI at −28 to −31 relative to the homeodomain.

During larval development, *msx3-Am* is specifically expressed in the ectoderm, but does not appear to be restricted to specific cell types (Figs. 4C–E). In pre-settlement stages, *msx3-Am* is expressed in the ectoderm covering approximately two-thirds of the embryo from the oral end, leaving one-third of the aboral end free of expression (Figs. 4C, E). Expression appears to be absent in the oral pore/gastric cavity. After settlement, *msx3-Am* is expressed in the ectoderm on the oral surface (Figs. 4F–I) but the transcript appears to be absent from the aboral surface (Figs. 4F, H). Expression appears to be concentrated in the upper part of the polyp as it grows upward (Fig. 4I).

The Acropora Pax-3/7 gene, Pax-Dam

We have previously reported the *Pax-Dam* sequence (Miller et al., 2000); a corresponding *Nematostella* sequence is in the databases as AAW29069. Whereas Pax-2/5/8/6-type genes have been cloned from a number of cnidarians (the homeodomains encoded by two of the three other known *Acropora* Pax genes are included in Figs. 5A and B), the anthozoan PaxD genes represent the only Pax-3/7-related genes thus far cloned from any cnidarian.

Pax-Dam hybridization was not detected in 15 h (prawn chip-stage) or 24 h (donut-stage) embryos despite indications from northern analysis that the gene was expressed (albeit at low levels) at these times. The first detectable *Pax-Dam* expression appears in a band near the aboral end of early planulae (Fig. 5C) and persists until late in planula development (Fig. 5D). As in the case of *cnox-2Am* (Hayward et al., 2001), *Pax-Dam* expression is completely excluded from the ectoderm at the aboral extremity of developing coral larvae. Expression of *Pax-Dam* is not restricted to distinguishable cell-types; rather, as in the cases of *msx3-Am* and the vnd genes, all cells in the delimited region are stained. On settlement there is a remarkable change in the expression pattern, with expression switching from the aboral to the oral end (compare Figs. 5C and D with Figs. 5E and F). As the polyp begins to grow upward from the basal calcified platform that it has laid down, there is expression in the ectoderm and along the medial portion of each septum (Fig. 5G).

Discussion

Although homologs of the genes which we investigated are widely considered to be definitive markers of the two axes of bilaterians, larval expression of the *Acropora* genes is restricted only along the primary (O/A) body axis, with no evidence for differences in transcript levels across secondary axes (see data summarized in Fig. 6). However, post-settlement expression of the *vnd* genes is in a bilateral or biradiate pattern. Expression of each of the gene classes studied here is restricted to the ectoderm during the pre-settlement stages of *Acropora* development. As in the case of *cnox-2Am* (the ind homolog), *emx-Am* is expressed predominantly or exclusively in putative neurons. *otxA-Am* is expressed in an otherwise uncharacterized type of ectodermal cell, while *msx3-Am* and *Pax-Dam* are more generally expressed. During larval development, the expression

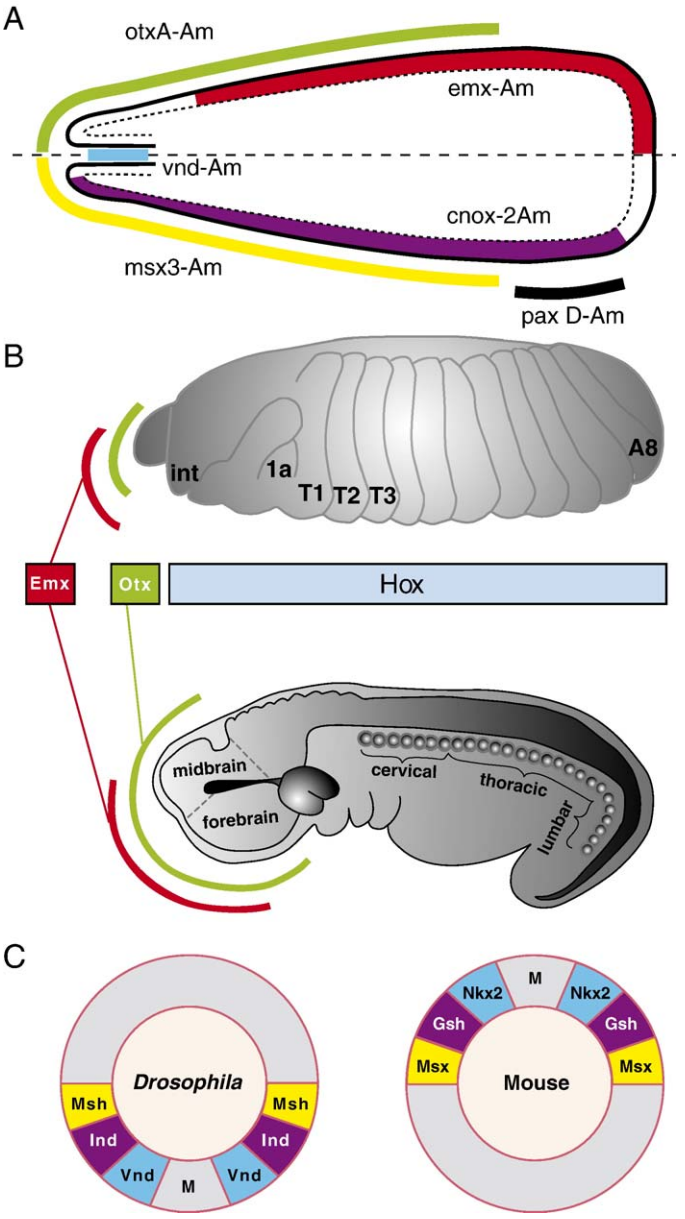


Fig. 6. Diagrammatic comparison of the zones of expression of the *Acropora* genes discussed in this paper (A) with the major A/P patterning genes of fly and mouse (B) and the genes that pattern the fly and mouse nervous systems in the D/V plane (C). (A) Either uniquely or in combination, the quite limited group of homeobox genes coding for transcription factors discussed here gives almost all parts of the *Acropora* planula a unique molecular address. For simplicity, the expression patterns of the *Acropora* homologs of the head gap genes (Otx/otd and Emx/ems) are shown in the upper part only, and those of the dorso-ventral regulators (NK2.1/vnd, Gsh/ind, Msx/msh and Pax-3/7) in the lower part only, although in all cases expression is symmetrical about the horizontal line. Despite being clearly related to genes that play key roles in anterior patterning in both *Drosophila* and vertebrates, *otxA-Am* and *emx-Am* are expressed at opposite ends of the larva. (B) Diagrammatic summary of the zones of expression of the major genes patterning the A/P axis of fly and mouse. (C) Idealized cross-sectional diagrams of the pre-gastrulation *Drosophila* and the neural tube of the mouse, showing the expression patterns of the genes that pattern the D/V axis of the nervous system of the respective species. Parts B and C modified from Ball et al. (2004).

of the vnd genes is limited to the pharyngeal ectoderm, which appears to consist exclusively of gland cells, but it is not clear what cell types express these genes in post-settlement material.

Comparison with anthozoan Hox-like genes

In terms of understanding the nature of axial patterning in anthozoans, the most directly relevant data to those presented here are for Hox-like genes (Finnerty et al., 2004), and dorso-ventral genes (Matus et al., 2006) in *Nematostella*. Like *msx3-Am*, *Pax-Dam* and the *Acropora* vnd genes, *Nematostella anthox1* is expressed in the larval ectoderm in an axially restricted manner, with transcripts detected only at the aboral extremity. Although it has been argued that *anthox1* is a posterior Hox gene (Finnerty et al., 2004), it appears to be a cnidarian-specific type that is equally similar to intermediate and posterior Hox classes (Kamm et al., 2006). Moreover, only one of the five published *Nematostella* Hox-like genes can be considered a member of a true Hox class—the other four genes are paralogous pairs (*anthox1* and *anthox1a*; *anthox7* and *anthox8*) that most likely arose within the Cnidaria (Kamm et al., 2006). *anthox6* is an anterior-Hox-like gene (Kamm et al., 2006) but is expressed at the oral extremity (Finnerty et al., 2004) which is the posterior end with respect to swimming direction and the opposite end of the planula to a definitive *emx* gene in *Acropora* (Fig. 6). Although the *Nematostella anthox6* gene is expressed orally, its likely orthologs in the hydrozoans *Eleutheria* (*Cnox-5ed*; Kamm et al., 2006) and *Podocoryne* (*Cnox-1pc*; Aerne et al., 1995) are expressed at the opposite (aboral) end of planulae (i.e. the front end with respect to swimming direction). The other characterized *Nematostella* Hox-like genes are expressed exclusively in the endoderm, in the central region along the O/A axis but asymmetrically with respect to the secondary axis. Hence, although some of the cnidarian Hox-like genes may fulfil analogous (i.e. non-homologous) roles, the Hox paradigm appears to be of limited value in understanding the basis of O/A patterning in anthozoans and its relation to A/P patterning in Bilateria. However, the gene expression patterns in *Acropora* summarized in Fig. 6 are reminiscent of Hox patterns in Bilateria to the extent that their zones of expression have an axis-patterning potential, suggesting the possibility that in *Acropora*, homeobox genes with different DNA-binding specificities may fulfil roles analogous to bilaterian Hox genes.

Evolution of axial patterning systems

Clearly no simple relationship exists between the major longitudinal axes of the Cnidaria and Bilateria, since components of both the A/P and D/V systems of the latter are differentially expressed along the same primary axis of the coral larva. The *Acropora* homologs of the three D/V columnar genes (*msx3-Am*, *cnox-2Am* and the three vnd genes) show axially restricted expression in the ectoderm, but direct cross-repressive interactions of the type known from *Drosophila* are unlikely to occur. The expression patterns of *msx3-Am* and *cnox-2Am* overlap substantially but not completely, with no sign of a step in intensity

of expression at the margin of overlap to suggest that the genes interact. vnd-Am transcripts are restricted to the pharyngeal ectoderm lining the mouth, which appears to consist exclusively of gland cells (Ball et al., 2002). So the apparently discrete expression domains of vnd and cnox2 may simply reflect the apparent absence of neurons in the oral ectoderm, consistent with the apparent lack of RFamide expressing cells in that area.

The simplest rationalization of the developmental expression data presented here and summarized above for other systems is that, as suggested by Hobmayer et al. (2000), at least some parts of both axis-specification systems known from the Bilateria coexisted in the common ancestor of modern cnidarians and bilaterians but reached their present separation and sophistication in the latter only after divergence. However, expression data for Dpp/BMP4 homologs in *Acropora* (Hayward et al., 2002 and unpublished) and *Nematostella* (Finnerty et al., 2004) mark a second axis during anthozoan development, as do those of *NvBMP5-8* and *NvChordin* in *Nematostella* (Matus et al., 2006). Although Shh is also essential to ventral neural tube patterning, Dpp/BMP4 signaling directly regulates Msx/msh in both vertebrates and *Drosophila* (Liem et al., 1995; Oh et al., 2002) and can also repress vnd when ectopically expressed (Oh et al., 2002). Moreover, a Dpp/BMP4-response element in the murine Msx2 promoter appears to have been conserved between mammals and *Drosophila* (Brugger et al., 2004), indicating an ancient role for Dpp/BMP4 signaling in regulating D/V patterning across the Bilateria. One major difference between the Bilateria and Cnidaria may therefore be that the latter diverged before Msx/msh and other D/V patterning genes came under regulation by Dpp/BMP4. Whereas their larval expression patterns show no hints of asymmetry in secondary axes, after settlement the *Acropora* vnd genes are expressed asymmetrically in a bilateral or bi-radiate pattern in the ectoderm around the oral pore. However, expression data for Dpp/BMP4 (Ball et al., unpublished) seemingly preclude a direct involvement in regulation of the *Acropora* vnd genes either pre- or post-settlement. Understanding the regulation of axial genes in cnidarians will require a thorough analysis of the corresponding cell–cell signaling systems, including the wnt, TGFb and Ras/MAPK families. Wnt signaling is known to repress the vertebrate homologs of several of the homeobox genes studied here, Emx1, Emx2 and Gsh2 (Backman et al., 2005) and Msx1 and Pax-3 (Monsoro-Burq et al., 2005), and clearly also plays a role in patterning the primary (O/A) axis in *Hydra* polyps (Hobmayer et al., 2000, Broun et al., 2005) and in *Nematostella* (Hobmayer et al., 2000; Wikramanayake et al., 2003; Kusserow et al., 2005). Whereas the wnts appear to function primarily in patterning the O/A axis, the TGFb ligands Dpp/BMP4 (Hayward et al., 2002; Finnerty et al., 2004; Matus et al., 2006) and GDF5 (Finnerty et al., 2004) are expressed in asymmetric patterns in a second axis. Several of the Hox-like genes are likewise expressed differentially across the secondary axis during *Nematostella* development (Finnerty et al., 2004), therefore roles for Dpp/BMP4 cannot be ruled out in these cases as Dpp/BMP4 regulates many of the Hox genes in both *Drosophila* (Marty et al., 2001, Grienenberger et al., 2003) and vertebrates (Shi et al., 1999; Williams et al., 2005).

The diversity of both transcription factors and signaling pathways employed during early development, and the complexity of their expression patterns, are remarkable given the morphological simplicity of anthozoans. The presence of FGF ligands (Technau et al., 2005) and hedgehog pathway components (Hooper, Hayward and Ball, unpublished) suggests that, as in vertebrates, these pathways may also play critical roles in early anthozoan development. The identification of targets of Dpp/BMP4 signaling in *Acropora* and *Nematostella*, and the evaluation of Hox-like genes as candidates in this respect, represent promising leads for understanding gene interactions in Urbilateria and how the present complex gene networks of Bilateria may have evolved.

Note added in proof

Between submission and publication of this paper, the Technau group published a paper that comes to very similar conclusions to those outlined above, but based on a different set of genes. On the basis of molecular interactions between BMP-like molecules and their secreted antagonists Rentzsch et al. (2006; Dev Biol. 296, 375-87) conclude ”that there is no simple relationship between the oral-aboral body axis of Nematostella and one particular body axis of Bilateria.”

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.07.034.

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